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Original article

Chitosan oligosaccharides protect nucleus pulposus cells from hydrogen peroxide-induced apoptosis in a rat experimental model

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ABSTRACT

Chitosan has been investigated for its protective effect on nucleus pulposus (NP) cells against intervertebral disc degeneration(IDD), but its high molecular weight prohibits its clinical application. The low molecular derivatives, chitosan oligosaccharides (COS) are easier to absorb; however, the protective effect of COS against IDD remains unclear. In this study, we investigated the effects of COS on NP degeneration. NP cells derived from rats were treated with H_2O_2 to induce an IDD-like transition. Then, COS was used to pre-treat cells before administering H_2O_2 and changes occurring in the cells were observed. As shown by the result of a cell counting kit-8(CCK-8) assay, COS protected the viability of the cells. The induced apoptosis rate fell when cells were pre-treated with COS, revealed by annexin-V FITC/PI double staining analysis and Hoechst 33342 staining. COS administration also protected ECM synthesis and prevented its degradation, as shown by western blotting(WB) and polymerase chain reaction(PCR). We analyzed the activity of the PI3K/Akt pathway in H_2O_2 treated NP cells by WB and the result showed that COS could enhance activity of the pathway. To investigate the relationship between the PI3K/Akt pathway and the protective effects of COS on NP cells, the PI3K/Akt pathway was inhibited by wortmannin, and we subsequently found that this abolished the protective effects. These results support the hypothesis that COS exerts its protective effect on NP cells against H_2O_2-induced apoptosis via the PI3K/Akt pathway.

1. Introduction

The intervertebral disc(IVD) is a significant component of the human spine, which gives the spine both stability and mobility. Intervertebral disc degeneration(IDD) is a continuous multistage process that can be detected during ageing or after generation of pathologic stresses, and can consequently cause serious pain. Severe IDD impairs the function of the IVD with a risk of causing IVD herniation [1]. IDD has been reported to be the cause of a large proportion of low back pain, a symptom which affects up to 80% of people at some point during their lives and can cause a heavy economic burden [2,3].

Although the precise mechanism underlying IDD is still unclear, research has discovered some of the changes which occur in IVD.

The IVD mainly consists of the nucleus pulposus(NP), the annulus fibrosus (AF) and the cartilage end plates (CEPs), and structural integrity of the IVD is the basis of its function [4,5]. The NP is a dynamic structure that maintains the function of the IVD and is thought to be the most important structure involved in the development of IDD [6,7]. Signs of IDD start to appear in the NPs once the bony elements of the spine mature during development of the body. Disappearance of NP cells shows up as the first sign of IDD [8]. At the same time, the chondrocytes of CEPs enter their hypertrophic conversion and start to synthesize type X collagen [9]. Then, the extracellular matrix(ECM) of the IVD becomes calcified and impermeable, which blocks both nutrient diffusion and metabolite removal of the NP cells [10]. Consequently the NP cells are exposed to metabolic stress, with consequence that the synthesis of enzymes that degrade ECM components including matrix metalloproteinases(MMPs) and A disintegrin and metalloproteinase with thrombospondin repeats(ADAMTS) become uncontrolled [11,12].

Previous studies have revealed that while the IVD is degenerating, the NP cells can initiate a series of anti-apoptotic and anti-senescence activities, such as expression of anti-apoptosis and anti-senescence genes and regulation of some signaling pathways, to protect the IVD [13].

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Chitosan and its derivatives are agents that have many biological effects [14,15]. Some derivatives of chitosan as well as chitosan itself have been proven to play a protective role in NP cells in IDD [16–19]. Due to its high molecular weight, chitosan cannot easily be absorbed by the body and its clinical application is thus limited. COS are a degradative product of chitosan, and show many similar effects [20] as well as being more easily absorbed [21]. Zhang et al. proved that COS has an anti-apoptotic effect on chondrocytes in osteoarthritis[22], thus the inhibitory effect of COS on chondrocyte apoptosis has been verified, but few studies have been conducted to investigate its effect on NP cells. We decided to investigate whether COS can protect NP cells against some pathological stimuli *in vitro*, with the aim of finding a new potential therapy for IDD.

2. Materials and methods

2.1. Animals and reagents

NP cell donators were healthy male Sprague-Dawley (SD) rats with an average weight of 364 ± 33 g. These rats were bought from Centre of Experimental Animals of Wuhan University, Wuhan, China. Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12), fetal bovine serum(FBS), trypsin, type II collagenase, and TRIzol were bought from Gibco BRL (Paisley, UK). Chitosan oligosaccharides (COS, MW <1 kDa; degree of deacetylation ≥93%) were from the Zhaoqing Longline Biotechnology Co., Ltd. (Guangdong, China). The cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Annexin- V-FITC/propidium iodide(PI) apoptosis detection kit was procured from BD Biosciences Pharmingen (USA). Hoechst 33342 was bought from Beyotime Institute of Biotechnology (Haimen, China). Phosphate-buffered saline (PBS, ×10, ST476) were bought from the Beyotime Institute of Biotechnology (Haimen, China) and were of highest purity commercially available. RIPA Lysis Buffer was purchased from Beyotime(Beijing, China). Rabbit polyclonal primary antibodies for mmp-13(Ab39012, Abcam), aggrecan(ab3778, Abcam), collagen type II (ab34712, Abcam), adamts-4(Eap1002, Elabscience), β-actin(ab8227, Abcam), total PI3K(#4292, Cell Signaling Technology), phospho-PI3K p85 (Tyr458)/p55 (Tyr199) (#4228, Cell Signaling Technology), total Akt(#9272, Cell Signaling Technology), Phospho-Akt (Ser473) (#9271, Cell Signaling Technology) were used. PI3K inhibitor wortmannin was bought from Cell Signaling Technolog(Beverly, MA, USA). An qRT-PCR kit was supplied by Invitrogen(Paisley, UK). Other chemicals and reagents were of high purity and commercially available.

2.2. Cell isolation and culture

NP cells were derived from 10-week-old male rats weighing 364 ± 33 g. The protocols were approved by the institutional ethics committee of Wuhan University School of Medicine. Firstly, pentobarbital (100 mg/kg body weight; Shanghai Biorui Biological Technological Co., Ltd., Shanghai, China) was used to euthanize the rats, and we derived lumber intervertebral discs from the spinal columns. Then, we separated NPs from AFs using an atomic microscope under aseptic condition. Immediately after separation, the NPs were digested by 0.1% type-2 collagenase (Gibco BRL) at 37 °C in a KYC-100C gyratory shaker(Shanghai Fuma Laboratory Instrument Company; Shanghai, China) at 110 rpm for 4 h. Supernatant was obtained after digestion and the cells were washed by DMEM/F-12 before being transferred into 25 cm^2 culture flasks. DMEM/F-12 with 15% FBS and a penicillin-streptomycin solution (SV30010; HyClone; 100 mg/ml streptomycin and 100U/ml penicillin) was used to incubate the cells in a 5% CO_2 incubator. The medium was refreshed every other day except the first change was conducted 6 days later immediately after the cells were planted into the plates. The NP cells were chondrocyte-like cells, which could be identified by type II collagen and aggrecan immunohistostaining.

2.3. Establishment of apoptotic models of NP cells

H_2O_2 (Wuhan Boster Biological Technology Company, Wuhan, China) was used to induce apoptotosis in NP cells. The second-passage NP cells (cell density of 1×10^6/ml) were cultured overnight before different concentrations of H_2O_2 (100, 200, 300 and 400 μM, respectively) were added into the medium. NP cells were examined at 0, 6, 12 and 24 h since H_2O_2 addition. To investigate the effects COS has on H_2O_2-treated NP cells, COS was added into the medium 2 h prior to H_2O_2. The concentrations of COS were 50 μg/ml, 100 μg/ml, 200 μg/ml, respectively.

2.4. Cell viability assay

CCK-8 assay was used for cell viability assessment. Cells were transferred into 96-well flatbottomed microplates at a final concentration of 2×10^4 cells per well in suspension. After 24 h incubation the medium was replaced with DMEM/F-12 containing H_2O_2, COS or phosphate-buffered saline (PBS; control group), respectively. Cells were treated with 100, 200, 300 and 400 μM H_2O_2, respectively, without COS, or 300 μM H_2O_2 along with different concentrations of chitosan oligosaccharides (50, 100 or 200 μg/ml). 10 μl CCK-8 reagents were added to each well containing 100 μl DMEM/F-12 to analyze the cell proliferation. Then the plate was incubated at 37° C for 2 h. The optical density was measured at light of a 450 nm wavelength using an EL × 800 Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). The cell viability calculating formula is as follows: Cell viability (% of control) = [(Ae − Ab)/(Ac − Ab)] × 100. Ae, Ab and Ac represent the A450 of the experimental, the blank and the control groups, respectively. Effect of COS on cells was also detected by the method described above. Experiments were performed in triplicate independently.

2.5. Annexin V-fluorescein isothiocyanate(FITC)/propidium iodide (PI) staining

Flow cytometric analysis was used to determine the level of apoptotic death in the NP cells. Cellular apoptosis was observed by annexin V-FITC/PI double staining, performed using an Annexin V/FITC Apoptosis Detection kit I (no. 556547; BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions.

Cells were seeded into 6 well plates in a concentration of 6×10^5/ml. After a 24 h treatment of H_2O_2, COS or a combined treatment, cells were trypsinized(Gibco-BRL, Rockville, MD, USA) and washed twice by PBS, centrifugated at 400 g for 5 min. Then, 500 μl binding buffer was used to re-suspend the cells. Again the cells were centrifugated and re-suspended by binding buffer. After re-suspension, cells were transferred into a flow cytometry glass tube, and stained with 5 μl annexin V-FITC/PI at room temperature. One hour later immediately after annexin V-FITC/PI were added, Cell Quest Pro software (version 4.01; BD Biosciences) in the BD FACSVerse™ flow cytometer (BD Biosciences) was then used to access the apoptosis rate under a light wavelength of 488 nm.

2.6. Nuclear staining with Hoechst 33342

Hoechst 33342 staining was used to assess apoptotic nuclear morphology. To determine whether COS protects cells from H_2O_2